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16. ABSTRACT

A new metabolite of chlorpyrifos was discovered in a human poisoning case in which a lethal quantity of the pesticide was ingested. The metabolite was isolated from a human liver extract. After extensive cleanup, the metabolite was subjected to various instrumental analyses such as gas chromatography, mass spectrometry, and nuclear magnetic resonance. The metabolite was identified as a compound similar to chlorpyrifos with a methylthio ($-SCH_3$) group substituted for a chlorine on the pyridinol ring. The method of isolation and the data obtained from the instrumental analyses are presented.

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should be possible to specifically breed cotton plants that contain the most effective insecticidal mixture of heliocides.

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JOURNAL ARTICLE

A New Metabolite of Chlorpyrifos: Isolation and Identification

Emile M. Lores,* G. Wayne Sovocool, Robert L. Harless, Nancy K. Wilson, and Robert F. Moseman

A new metabolite of chlorpyrifos was discovered in a human poisoning case in which a lethal quantity of the pesticide was ingested. The metabolite was isolated from a human liver extract. After extensive cleanup, the metabolite was subjected to various instrumental analyses such as gas chromatography, mass spectrometry, and nuclear magnetic resonance. The metabolite was identified as a compound similar to chlorpyrifos with a methylthio ($-SCH_3$) group substituted for a chlorine on the pyridinol ring. The method of isolation and the data obtained from the instrumental analyses are presented.

Pesticide residue analyses were performed on autopsy samples taken from an individual suspected of having accidentally ingested a pesticide formulation (Lores et al., 1978). The results of these analyses indicated that a mixture of chlorpyrifos [*O,O*-diethyl *O*-(3,5,6-trichloro-2-pyridyl) phosphorothioate] and malathion (diethyl mercaptosuccinate, S ester with *O,O*-dimethyl phosphorodithioate) had been ingested. During the analysis, a previously unreported metabolite of chlorpyrifos was detected in the liver.

This new metabolite was isolated from human liver. Gas chromatography (GC) with sulfur, phosphorus, and nitrogen specific detectors; combined gas chromatography–mass spectrometry (GC–MS); high-resolution mass spectrometry (HRMS); and nuclear magnetic resonance spectroscopy (NMR) were used to characterize the metabolite. It was identified as the *O,O*-diethyl phosphorothioate ester of a dichloro, methylthio, 2-pyridinol. The exact position of the methylthio ($-SCH_3$) group on the pyridine ring could not be determined; but one of the three possible positions was ruled out by proton NMR.

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EXPERIMENTAL SECTION

Gas Chromatography. All gas chromatographic analyses were carried out on a Tracor Model 222 GC. Two types of detectors were used. A Flame Photometric Detector (FPD) equipped with a Spectrum 1020 noise filter and a variable voltage power supply (Power Designs) was used to detect phosphorus or sulfur. The selectivity of the detector could be changed simply by changing the light filter between the flame and the phototube. A Hall electrolytic conductivity detector was used to selectively detect nitrogen.

Three columns were used in the gas chromatographic analyses: 5% OV-210 on Gas-Chrom Q 80–100 mesh; 4% SE-30/6% OV-210 on Gas-Chrom Q 80–100 mesh; and 1.5% OV-17/1.95% OV-210 on Gas-Chrom Q 80–100 mesh. All columns were operated at 200 °C with 40 mL/min N_2 carrier gas. The inlet was kept at 225 °C. The flame photometric detector was operated at 175 °C. The Hall detector furnace was operated at 860 °C.

Gas Chromatography–Mass Spectrometry. Initial 70-eV electron impact mass spectra were obtained from a Hewlett-Packard 5930A mass spectrometer equipped

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with a Model 5700A gas chromatograph. Ions were detected using a Bendix continuous dynode electron multiplier at 2.5 kV and recorded on a light beam oscillograph. A 183 cm × 2 mm (i.d.) glass column packed with 1.5% OV-17/1.95% OV-210 on 80–100 mesh Gas-Chrom Q was used. The GC inlet temperature was 200 °C. Helium flow rate was 42 mL/min over a membrane separator at 190 °C. Isothermal runs were made at 190 °C.

Chemical ionization mass spectra were obtained on a Finnigan 3200 mass spectrometer with a Model 6002 data system and a Model 9500 gas chromatograph. The GC column, containing the same packing as described above, was operated isothermally at 210 °C. Individual runs were made with methane and helium as the reagent gas maintained at 1 Torr in an ion source operated at 60 °C.

High-Resolution Mass Spectrometry. High-resolution mass spectrometric analyses were performed with a Varian MAT 311 Mass Spectrometer interfaced with a Model 2700 GC and a SS 100 data system. The sample was introduced into the mass spectrometer ion source by GC or direct insertion probe.

The high-resolution peak matching technique utilizing perfluorokerosene (PFK) as the reference compound was used to determine the elemental composition of specific masses in the metabolite mass spectra. The HRMS operating parameters were: 3 kV acceleration voltage; electron energy 70 eV; electron current 2 ma; 7000–8000 resolution; ion source temperature 220 °C. The slit separator and all connecting lines were maintained at 240 °C. The GC parameters were: injection port temperature, 240 °C; helium flow, 12 mL/min. The 183 cm × 3 mm (i.d.) glass column contained 2% Dexsil 410 on 80/100 mesh Chromosorb W and was programmed from 100 to 200 °C, ca. 5 °C/min.

Nuclear Magnetic Resonance Spectroscopy. Proton NMR spectra were obtained by pulse Fourier transform operation on a Varian XL-100-15 spectrometer with a Nicolet TT-100 Fourier transform system. Quadrature phase detection, 500 Hz spectral widths, and an 8K data table were used. The 90° pulse width was 16 μs and the flip angle used was 22°. The spectra were obtained at the probe ambient temperature, ca. 28 °C.

Samples of chlorpyrifos, its metabolite, and the GC column bleed were analyzed as solutions in acetone-d_6 with 0.5% tetramethylsilane as an internal reference. The samples were contained in 5-mm (o.d.) NMR tubes.

Method of Isolation. The new metabolite was first detected in an acetone extract of a 0.5 g autopsy sample of liver during an attempt to identify the toxic agent in a human poisoning case. GC-FPD analysis of the extract indicated a large chlorpyrifos peak and one unidentified peak with a retention time approximately 2.35 times that of chlorpyrifos (the unidentified peak could not be matched on the standard relative retention time charts of Thompson (1974)).

Samples of the chlorpyrifos metabolite were isolated three times. The first sample was used for GC analysis and a tentative GC-MS identification. The second sample was larger and was subjected to a more rigorous cleanup for GC-MS analysis. The third sample required a very large sample (approximately 200 g) and even more cleanup to obtain enough pure metabolite for proton NMR analysis.

To extract the first sample, 0.5 g of liver was homogenized with 0.5 mL of distilled water with a Kontes Duall Homogenizer (catalog No. K588545). Ten milliliters of acetone was added and the sample was homogenized again. The homogenate was centrifuged and the supernatant was transferred to a 15-mL glass-stoppered centrifuge tube. Five-microliter aliquots of the supernatant were injected on the GC-FPD (phosphorus and sulfur modes were used). The remaining supernatant was evaporated to approximately 1 mL and partitioned into hexane. The hexane was then evaporated to 0.1 mL. A 5-μL aliquot of this was injected on the GC with a nitrogen specific Hall electrolytic conductivity detector.

A second sample of 10 g was extracted for analysis by mass spectrometry. The 10-g sample was homogenized in a Polytron (Brinkman Instruments, Inc.) and extracted with acetone. The acetone extract was evaporated until only water remained. The remaining water was then extracted with small volumes of hexane (5 mL) until none of the metabolite could be seen when a 5-μL aliquot of the hexane extract was injected on the GC. The combined hexane extracts were then evaporated to a volume of 1 mL, and the sample was put on a 25 mm × 30 cm silica gel column. The column was then eluted with successive 50-mL fractions of the following solvents: hexane; 10% benzene in hexane; 20% benzene in hexane; 30% benzene in hexane; 40% benzene in hexane; 50% benzene in hexane; 60% benzene in hexane; 70% benzene in hexane; 80% benzene in hexane; 90% benzene in hexane; benzene.

Benzene was used as the eluting solvent until the metabolite could no longer be detected on the GC without concentration. All fractions containing the metabolite were combined and the solvent was evaporated to about 0.2 mL. The 25-μL aliquots of sample were then injected into the GC, and the metabolite was collected as it eluted from a 4% SE-30/6% OV-210 GC column at 200 °C. The collection was made by connecting a Pasteur pipet packed with silica gel and glass wool to the vent tube with a short piece of Teflon tubing and diverting the column effluent to the vent by way of a four-port Valco valve. The Pasteur pipet was then rinsed with benzene to elute the metabolite. The solvent was evaporated to a volume of 50 μL. This sample was then subjected to GC-MS and HRMS. GC-MS in EI mode was run first, then CI spectra were obtained. The rest of this sample was placed on the tip of a direct insertion probe and the solvent evaporated in a stream of nitrogen. The sample was then introduced into the ion source of a high-resolution mass spectrometer.

The third extraction, which was intended for NMR, involved the same procedure as the second extraction up through the silica gel cleanup. After the silica gel, the third sample was put through a gel permeation column as an additional cleanup before the GC step. The GC effluent was collected on a Pasteur pipet and the metabolite was eluted from the pipet with acetone. The acetone was evaporated to approximately 0.1 mL and 1.0 mL of deuterated acetone was added for NMR. The sample was dried over 3 Å molecular sieves and evaporated to approximately 0.3 mL for NMR analysis. After being used for NMR the sample was again subjected to HRMS.

RESULTS AND DISCUSSION

The metabolite was first detected by a GC with a Flame Photometric Detector operated in the phosphorus mode (Figure 1). This indicated the presence of a phosphorus compound similar in structure and behavior to known organophosphorus (OP) pesticides. A second analysis was run with the FPD in the sulfur mode. This test was positive and the response relative to chlorpyrifos, which was also present in the sample, was more than double the relative response on the phosphorus detector. This increase in relative response was the first indication of two sulfur atoms in the compound. The presence of nitrogen in the molecule was indicated by an injection on a GC with

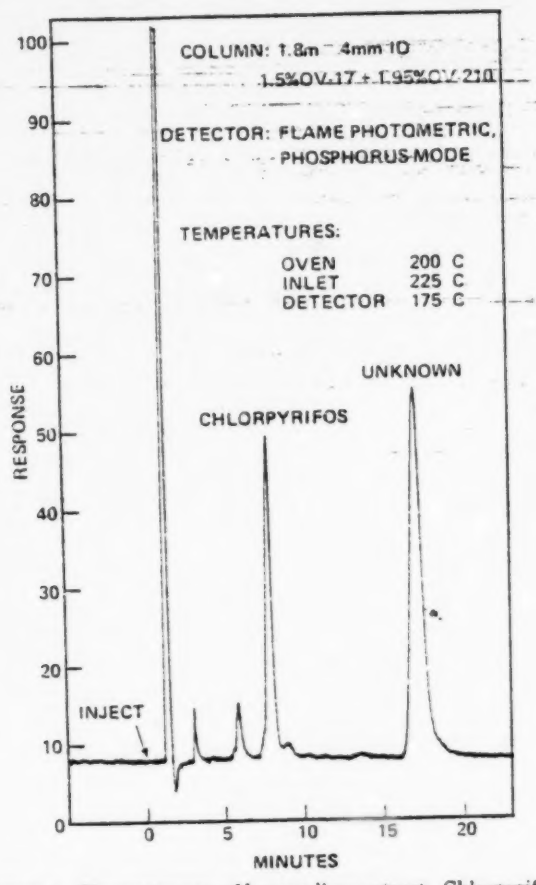


Figure 1. Chromatogram of human liver extract. Chlorpyrifos in this sample represents approximately 0.08 ppm.

Table I. Relative Retention Times (RRT) of Chlorpyrifos and Metabolite[a]

Column	RRT of chlorpyrifos	RRT of metabolite
5% OV-210	0.45	1.03
1.5% OV-17/1.95% OV-210	0.74	1.75
4% SE-30/6% OV-210	0.67	1.43

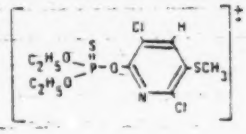
[a] All retention times relative to ethyl parathion.

a nitrogen specific Hall detector. The relative retention time (RRT), referenced to ethyl parathion, was determined on all three columns (Table I). An examination was made of the RRT charts for organophosphorus pesticides (Thompson, 1974). Since none of the compounds on the RRT charts matched the unknown on all three columns, additional analyses were required.

The retention time of the metabolite relative to ethyl parathion was used to determine the data acquisition point on the first GC–MS run. The total ion chromatogram was obscured by the background in the sample. However, electron impact spectra taken at the retention time of the unknown indicated a compound similar to chlorpyrifos, but with two chlorine atoms and a molecular ion at 12 atomic mass units (amu) higher than chlorpyrifos, as can be seen in Figure 2. The rest of the mass spectrum was similar to the mass spectrum of standard chlorpyrifos, except that most of the fragments in the mass spectrum were shifted 12 amu higher and contained one less chlorine. This seemed to indicate that a moiety with a mass of 47 amu had been substituted for one of the chlorpyrifos chlorines. Figure 2 shows the mass spectra of the metabolite and chlorpyrifos. The shift of the mass spectrum

TABLE II HIGH RESOLUTION MASS MEASUREMENTS

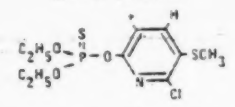
MOLECULAR ION CLUSTER



MOLECULAR FORMULA: $C_{10}H_{14}O_3S_2PN\ ^{35}Cl_2$
EXACT MASS: 360.9538
EXPERIMENTAL MASS: 360.9528

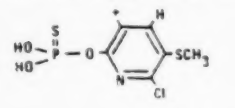
MOLECULAR FORMULA: $C_{10}H_{14}O_3S_2PN\ ^{35}Cl\ ^{37}Cl$
EXACT MASS: 362.9508
EXPERIMENTAL MASS: 362.9501

$M^{+\cdot} - Cl$ CLUSTER



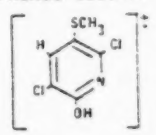
MOLECULAR FORMULA: $C_{10}H_{14}O_3S_2PN\ ^{35}Cl$
EXACT MASS: 325.9842
EXPERIMENTAL MASS: 325.9841

$M^{+\cdot} - [Cl + 2C_2H_4]$ CLUSTER



MOLECULAR FORMULA: $C_6H_8O_3S_2PN\ ^{35}Cl$
EXACT MASS: 268.9215
EXPERIMENTAL MASS: 268.9215

"PHENOL" CLUSTER



MOLECULAR FORMULA: $C_6H_5\ ^{35}Cl_2SON$
EXACT MASS: 208.9469
EXPERIMENTAL MASS: 208.9470

to 12 amu higher can be seen for all fragments that contain the pyridinol ring. This also indicated that the substitution had occurred on the ring, not on another part of the molecule. One of the few functional groups with a mass of 47 amu is a thiomethyl ($-SCH_3$) group.

The presence of intense fragments at 97 and 125 amu is consistent with the *O,O*-diethyl phosphorothioate part of the molecule remaining unchanged (Waller, 1972). Chemical ionization spectra were run and confirmed the molecular ion at 361 amu.

To confirm the proposed molecular formula, HRMS was employed. All of the HRMS measurements were within 1 ppm of the proposed compositions of the molecular and fragment ions, as can be seen in Table II.

Next, NMR was used to try to determine the exact position of the thiomethyl group on the pyridine ring. Chlorpyrifos has only one ring proton. By measuring the chemical shift of that proton on the metabolite relative to its chemical shift in chlorpyrifos, the ortho or meta relationship to the thiomethyl group can be determined.

Comparisons of data for a number of model compounds (Jackman, 1969) indicate that substitution of a thiomethyl for a chlorine on an aromatic ring should give a large increase in shielding of a proton ortho to the SCH_3, and a decrease or small increase in shielding of a proton meta to the SCH_3. In the closely related group of pesticide compounds, bromophos, ethyl bromophos, ronnel, and chlorthiophos, this SCH_3 substituent effect can be more closely evaluated. The replacement of a chlorine (in ronnel) by a thiomethyl (in chlorthiophos), after correction for the change of alkyl substituents on the phosphate (obtained from the bromophos and ethyl bromophos data), gives a large increase in shielding of the proton ortho to the SCH_3, −0.420 ppm, and a much smaller increase in shielding of the proton meta to the SCH_3, −0.127 ppm. In chlorpyrifos, the chemical shift of the ring proton is δ 8.313. In the metabolite, this chemical shift is δ 7.95, which corresponds to an increase in shielding of −0.36 ppm, and

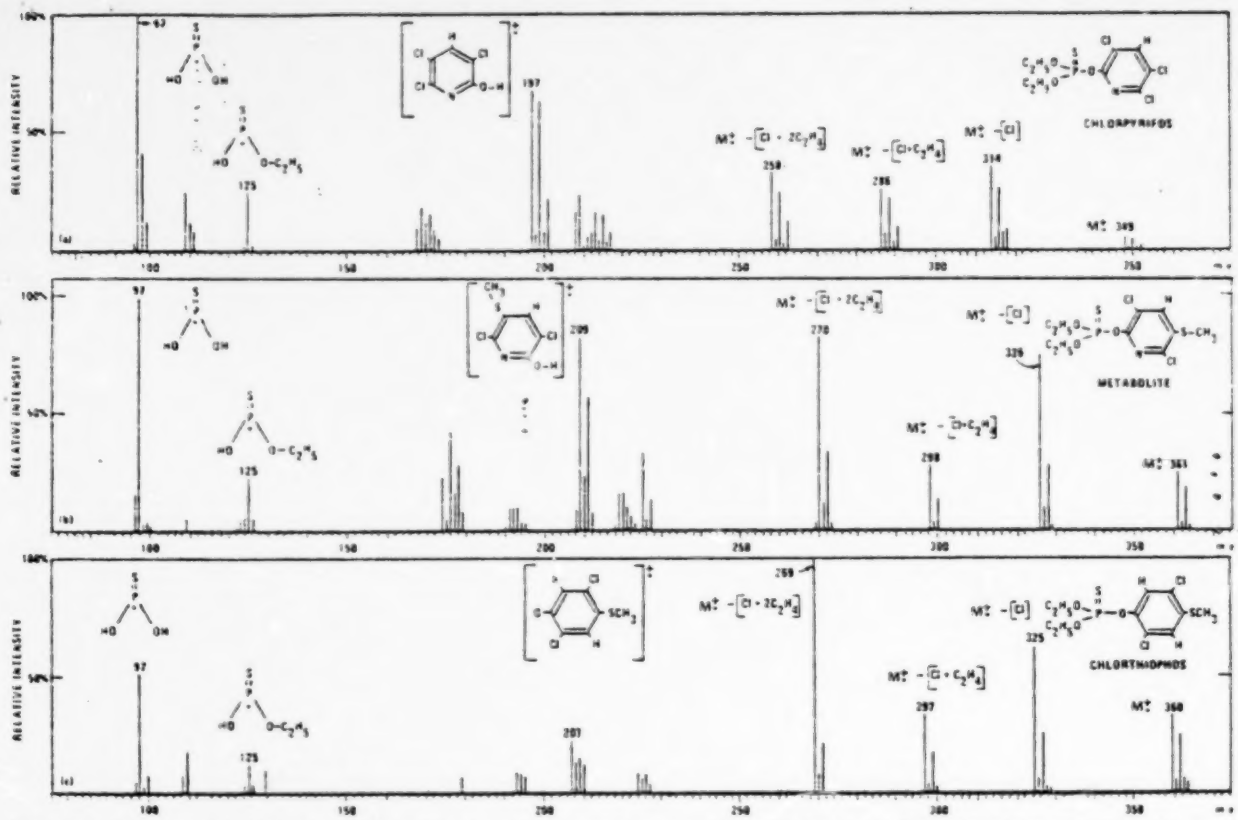


Figure 2. Mass spectra of the metabolite and related compounds run on a Hewlett Packard 5930A GC–MS in EI mode: (a) mass spectrum of chlorpyrifos, (b) mass spectrum of the metabolite, (c) mass spectrum of chlorthiophos, a pesticide similar to the metabolite.

clearly indicates that the thiomethyl group is ortho to the ring proton. Thus, there are only two possible positions for the thiomethyl group, ring positions number 3 and 5. The exact position of the thiomethyl group could not be determined from the data available.

A sample of the ingested formulation was available and was analyzed for the metabolite. This analysis was negative and eliminated the possibility of a nonmetabolic origin of this compound.

The presence of this metabolite in this one poisoning case does not necessarily indicate a major metabolic pathway in humans. Since this case involved a person with cirrhosis of the liver and carcinoma of the tongue, an unusual pathway may have been induced by a drug or disease. Also, the presence of the malathion or the large dose received could have induced a secondary metabolic pathway.

Metabolism of this type has been reported. The metabolism of pentachloronitrobenzene to pentachlorothioanisole, where the $-NO_2$ group was replaced by $-SCH_3$, in dogs, rats, cows, plants, fungi, and soil has been reported (Kuchar et al., 1969; Nakanishi and Oku, 1969; Borzelleca et al., 1971; DeVos et al., 1974). Koss et al. (1976) reported finding pentachlorothiophenol in metabolism studies with hexachlorobenzene; however, the method used in that analysis would not distinguish pentachlorothiophenol from pentachlorothioanisole.

As for the metabolism of chlorpyrifos, Hutacharern and Knowles (1975) reported finding dechlorinated metabolites in subterranean termites. They also reported finding unidentified radioactive metabolites of ring-labeled [^{14}C]chlorpyrifos. Smith et al. (1967) reported finding a minor component in tissues of rats fed [^{36}Cl]chlorpyrifos. This minor component matched the elimination rate of [^{36}Cl]chloride and may have been formed by a dechlorination type metabolism.

The toxicity of the metabolite reported in this study is not known. However, a comparison of organophosphorus pesticides with similar functional groups by Eto (1974) shows that compounds containing a thiomethyl group ($-SCH_3$) are more toxic than those with a nitro or halogen substituent. This possibility of increased toxicity of this new metabolite needs investigation. Further structural identification and synthesis of this new metabolite, along with studies of the metabolic pathways and conditions that may alter the levels of the metabolite, especially in humans, are also needed.

SUMMARY

The isolation of a new metabolite of chlorpyrifos as a major component in human liver extract has been described. The identification of the metabolite as a thiomethyl derivative of chlorpyrifos has been established using gas chromatography with element specific detectors, gas chromatography–mass spectrometry, high-resolution mass spectrometry, and nuclear magnetic resonance. The exact ring position could not be determined but one of the three possible positions has been ruled out.

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Method for Extraction, Isolation, and Detection of Free Polybrominated Biphenyls (PBBs) from Plasma, Feces, Milk, and Bile Using Disposable Glassware

Lynn B. Willett,* Constance J. Brumm, and Cynthia L. Williams

A rapid method was developed for the extraction, isolation, and detection of polybrominated biphenyls (PBBs) from plasma, feces, milk, and bile, using disposable glassware. Use of disposable equipment greatly reduced the amount of laboratory background and cross-contamination of samples. The procedure employed a multiple extraction with a mixture of diethyl and petroleum ethers, followed by cleanup on miniature Florisil, silica gel, and sodium sulfate columns. Detection was accomplished by gas chromatography. Recoveries were determined for the six major components of a commercial PBB mixture and were approximately 96% for plasma, 59% for feces, and 98% for milk. The background levels for plasma, feces, and milk were 0.0005, 0.0007, and 0.0007 ppm, respectively, bringing the minimum detectable limits of the major hexabromobiphenyl peak to 0.0010, 0.0014, and 0.0014 ppm on a whole tissue basis.

Polybrominated biphenyls (PBBs) were manufactured for use as a fire retardant from 1970 to 1973 by the Michigan Chemical Corporation (St. Louis, Mich.) and marketed both as fireMaster BP-6 and as fireMaster FF-1. In the summer of 1973, fireMaster FF-1 was accidentally mixed with dairy feed in place of the supplement magnesium oxide, or Nutrimaster, resulting in the contamination of about 30 Michigan dairy farms (Whitehead, 1975). Many animals which had ingested this contaminated feed attained PBB levels as high as 300 ppm on a fat basis (Jackson and Halbert, 1974). After the original contamination the PBB compound persisted in feed manufacturing, storage, and handling equipment. As other feed passed through the mills and was cross-contaminated, many more farms acquired relatively low levels of PBB contamination (Dunckel, 1975). Regulatory agencies have destroyed vast numbers of livestock and food products because of contamination, thus eliminating highly contaminated animals and products from the food chain. The present Food and Drug Administration guideline tolerance levels of PBB are 0.3 ppm in milk and meat on a fat basis and 0.05 ppm in whole eggs (Dunckel, 1975). These levels are largely based on the degree of sensitivity of available methods of analysis for the compound (Kolbye, 1975). PBB levels below tolerance have been detected in food products. Legislation in Michigan has been initiated to further reduce the tolerance to 0.02 ppm. Since these guideline levels are low, it will be necessary to have an extraction method that is rapid, reliable, sensitive, and relatively free of background contamination to accurately quantitate low levels of PBB.

OBJECTIVE

The extraction, isolation, and detection method described here was developed during a controlled study of the toxicity in pregnant Holstein heifers which were fed fireMaster BP-6 at doses equivalent to 0 to 5000 ppm in their rations. Twenty-four animals were under observation with frequent sampling of blood, feces, and milk, resulting in a vast number of samples to be analyzed. A method for the analysis of these samples had to be developed that was both rapid and reliable throughout the wide range of concentrations.

Use of the standard method for the extraction and cleanup of halogenated hydrocarbons (USDHEW, 1971) was impractical and unusable because of the time and large amount of glassware necessary for each sample. Once samples containing high levels of PBB were passed through the glassware, it could no longer be used in the extraction and cleanup of low level samples. Control of background was impossible despite exhaustive cleaning and decontamination procedures. Methods which had proven to be effective for decontamination of PCBs in this laboratory were not effective for PBBs. It was apparent that all glassware needed to be disposable to prevent high background and cross-contamination.

METHOD

A method was developed for the extraction, isolation, and quantitation of PBBs from plasma, feces, milk and bile. All solvents were reagent grade and glass redistilled. Solvents concentrated 90% produced no gas chroma-

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